IMPERIAL BUREAU OF ANIMAL NUTRITION

Technical Communication No. 9

METHODS FOR THE CHEMICAL ANALYSIS OF BIOLOGICAL MATERIAL IN NUTRITION INVESTIGATIONS

EDITED BY

WILLIAM GODDEN

Head, Biochemical Dept., Rowett Research Institute, Aberdeen

IMPERIAL BUREAU OF ANIMAL NUTRITION

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Commonwealth Bureau of Animal Nutrition

FOREWORD.

THIS Bureau receives frequent enquiries from Oversea workers for detailed information on the ordinary chemical methods used in analysis of biological materials (foodstuffs, blood and the like). So frequent have these enquiries become that it seemed desirable to issue a bulletin setting out a series of methods suitable for general use. The Bureau sought the co-operation of Mr. W. Godden, Head of the Biochemical Department of the Rowett Research Institute, Aberdeen, in the publication of a Technical Communication for this purpose.

It was felt that such a Communication would be of undoubted value to the increasing number of workers throughout the Empire who are engaged in the study of nutritional problems. Many of these, owing to geographical location, do not have ready access to suitable libraries where journals and books of reference may be consulted. Further, it was thought that such a Communication might lead to a greater uniformity in analytical technique employed and thus ensure that data giving the composition of biological materials obtained in different parts of the Empire would be more strictly comparable.

As a basis for the proposed publication it was decided to use the methods adopted at the Rowett Research Institute. These have, in many cases, been modified to meet the requirements of the particular type of work in question, and are known to be used by a considerable number of workers in Empire countries who have at one time or another been attached to the Institute for study. It is by no means suggested that the methods employed at the Rowett Institute are of necessity the only suitable or reliable methods ; nor is it claimed that these methods have in every case originated from the Rowett Institute. They are, however, in most cases, methods which, after considerable experience, have been adopted as being the most satisfactory for general use. It is not intended that the compilation should constitute a text book of biochemical methods in their entirety, but rather that it should serve as a useful handbook principally for those engaged in human and animal nutrition studies.

Before proceeding with this publication, the Bureau consulted certain representative Empire workers, to whom a description of the methods was circulated for criticism, comments and suggestions. The undernoted kindly collaborated in this way, and in some cases submitted additional notes from other workers in their own country.

England and Wales.

Dr. H. H. Green, Ministry of Agriculture and Fisheries, Veterinary Laboratory, Weybridge, Surrey.

Northern Ireland.

Professor R. G. Baskett, Ministry of Agriculture for Northern Ireland, Chemical and Animal Nutrition Division, Queen's University, Belfast.

Dominion of Canada.

Mr. C. H. Robinson, Dominion Agricultural Chemist, Central Experimental Farm, Ottawa.

Commonwealth of Australia.

Mr. H. R. Marston, Nutrition Laboratory, Division of Animal Health and Nutrition (C.S.I.R.), University of Adelaide.

Dominion of New Zealand.

Mr. B. C. Aston, Chief Chemist, Department of Agriculture, Wellington.

Union of South Africa.

Dr. A. I. Malan, Division of Veterinary Services, Union Department of Agriculture, Onderstepoort.

India.

Dr. W. R. Aykroyd, Director, Nutrition Research Institute, Coonoor.

Dr. K. C. Sen, Physiological Chemist, Imperial Institute of Animal Husbandry and Dairying, Bangalore.

Southern Rhodesia.

Mr. A. D. Husband, Chief Chemist, Department of Agriculture and Lands, Salisbury.

By certain of these collaborators minor alterations in technique have been suggested. Others recommended alternative methods, and in addition, some correspondents have recommended the inclusion of methods for certain elements which were not dealt with among the selected methods originally circulated.

These replies, wherever it was considered desirable from the nature of and the ideas underlying the compilation of this Communication,

have been dealt with either by the addition of footnotes to the Rowett methods or by printing *in extenso* new methods. Due acknowledgment is made in every case. It should be realised that it was not feasible to include all minor alterations or alternatives, and in some cases, from the experience of workers at the Rowett Institute, it was felt that the alterations did not make for increased accuracy or ease of work.

It might be pointed out that the methods described do not cover the estimation of proximate principles, *e.g.* protein and fat. These latter methods have been for so long in the hands of analysts that reference to them was deemed unnecessary. Neither is the subject of vitamin assays dealt with, as this branch of work is highly specialised.

It is hoped that workers who, after a trial of these methods, have any criticisms or comments to make will not hesitate to forward them, so that any succeeding issues may be brought up to date.

CONTENTS.

PAGE

FOREWORD 3

I. ANALYSIS OF FOODSTUFFS 9

General Remarks 9

Calcium 10

Magnesium 11

Sodium and Potassium 12

Iron 15

Copper 17

Manganese 19

Cobalt 21

Zinc 23

Phosphorus 25

Chlorine 27

Iodine 28

Sulphur 33

II. ANALYSIS OF BLOOD 35

General Remarks 35

Serum Calcium 36

Serum Magnesium 37

Alternative Technique for Serum Calcium and Magnesium . 39

Inorganic Phosphorus 43

Plasma Phosphatase (Kay) 46

Serum Phosphatase (Bodansky) 47

Protein Precipitation 50

Chlorides 51

Sugar (Hagedorn and Jensen) 52

Sugar (Shaffer-Hartmann) 53

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I. ANALYSIS OF FOODSTUFFS.

GENERAL REMARKS.

(*Incorporating suggestions from various workers.*)

Designation of Sample.—In addition to naming the material correctly (both botanical and common name to be given where possible), notes on the nature of the material should be given. This is particularly desirable in the case of fodders, *e.g.* hay and dried grass, where the stage of growth and the method of making and storage should be indicated. Information regarding the district where the material was grown, the nature of the soil, manurial treatment and climatic conditions may also be of value.

Method of Sampling.—It is impossible in a few lines to describe appropriate methods of sampling for every type of foodstuff. These are, as a rule, laid down in most official regulations under Fertiliser and Feeding Stuffs Acts. Suffice it to say here that the sample should be representative of a large bulk of material and the particular procedure adopted in drawing the sample should be indicated. Avoidance of soil contamination should be specially guarded against.

Preparation for Analysis.—Wherever possible, if the material can be got to the laboratory quickly in air-tight containers, moisture should be determined on the original material. The material may then be air-dried or dried at a low temperature in a suitable oven and finely ground and thoroughly mixed. Whenever samples are being weighed out for subsequent analysis, samples should be weighed out for moisture determination so that all results may be calculated to a dry matter basis (oven-dried).

Expression of Results.—For the sake of uniformity, and also to conform with the now generally accepted mode of expression in human nutrition investigations, results should be expressed as percentages of the element and given as a percentage on the oven-dried dry matter. If it has been possible in the case of a crop to determine moisture on

the original material then the results can, if desired, be calculated back to the standing crop basis.

ESTIMATION OF CALCIUM IN FOODSTUFFS.

Method.

A sufficient quantity * of material is ashed to a grey ash in a vitreosil basin. The ash when cold is extracted twice with hot 2*N* HCl, and is then well washed with hot water in the usual manner. The filtrate and washings are made up to 250 ml. An aliquot * portion is taken in a 400 ml. beaker, diluted to about 200 ml. with distilled water, made just alkaline to litmus with 25 % ammonium hydroxide and then just acid with 10 % acetic acid.† To this solution, when boiling, 10 ml. of a boiling saturated solution of ammonium oxalate are added with stirring. The beaker is placed on a hot water-bath for about an hour for the precipitate to settle and then left overnight. The precipitate is filtered through a 9 cm. ashless filter paper (Whatman No. 40) and washed, first by decantation with hot water, until free from ammonium oxalate (four washings usually suffice). The filter paper and precipitate are transferred back to the beaker. Approximately 200 ml. distilled water and 10 ml. concentrated H_2SO_4 are added, the latter with stirring, and the mixture heated to 70° C. and titrated with *N*/10 $KMnO_4$.

1 ml. *N*/10 $KMnO_4$ ≡ 0·0020 g. Ca.

Notes from Oversea Workers.

Stone (S. Rhodesia). If solid ammonium oxalate is added, as precipitant, to the boiling solution the precipitate settles almost immediately.

* A quantity such as will give a titration figure of 10–15 ml. The amount of the aliquot will depend upon the weight of substance taken.

† In some cases there is a precipitate at this stage which usually clears up when the ammonium oxalate is added. If there is much aluminium present the precipitate may not disappear, and in this case the precipitate of oxalate must be redissolved after filtering and washing, and then reprecipitated, going through the procedure as above.

Alternatively, if there is a fairly bulky precipitate after acidifying with acetic acid, it may be filtered off and washed with hot water and the calcium precipitated in the combined filtrate and washings.

Malan (S. Africa). Recommends micro-Ca determination as under serum-Ca.

Baskett (N. Ireland). Considers there may be a risk in washing the precipitate with very hot water where only a small amount of Ca is present and a high degree of accuracy is sought.

ESTIMATION OF MAGNESIUM IN FOODSTUFFS.

Reference.

Official and Tentative Methods of Analyses of the Association of Official Agricultural Chemists. Washington, 1935 (fourth edition), page 124.

Method.

To the combined filtrate and washings from the calcium determination add 30 ml. HNO_3 and evaporate to dryness to decompose the ammonium salts. Take up with 5 ml. HCl and dilute to about 100 ml. with water. Add 5 ml. of a 10 % solution of sodium citrate and 10 ml. of a 10 % solution of ammonium phosphate, or enough to precipitate all the magnesium. Add ammonia (1 to 4 of water) with constant stirring (using a rubber policeman) until the solution is faintly alkaline and the precipitate forms ; then add about 5 ml. of strong ammonia, stir vigorously until the precipitate is granular, and allow to stand in a cool place overnight. Filter and wash free from chlorides with cold dilute ammonia (1 to 10). Dry, ignite and weigh as $Mg_2P_2O_7$.

Note from Oversea Worker.

Carbery (India). Recommends the above method, but states that in cases where the quantities of iron, alumina, etc., are large the amount of citrate to be added has to be increased. Equally satisfactory results are obtained by the addition of citric acid followed by ammonia as with sodium citrate as recommended.

ESTIMATION OF SODIUM AND POTASSIUM IN FOODSTUFFS.

Reference.

Husband, A. D. and Godden, W., *Analyst*, 1927, **52**, 72.

Reagents.

Alcoholic ammonium carbonate (Gooch and Eddy, *Zeit. anorg. Chem.*, 1908, **58**, 427): 180 ml. strong ammonia (sp. gr. 0·880) are diluted to one litre with water. One litre of 90 % alcohol is added, and in this mixture 250 g. powdered ammonium carbonate are dissolved by warming on a water-bath under an air reflux. When cold the solution is filtered and is ready for use.

Cobaltinitrite reagent (Adie and Wood, *J. Chem. Soc.*, 1900, 1076): 113 g. cobalt acetate are dissolved in 400 ml. water and 100 ml. glacial acetic acid are added. 200 g. sodium nitrite are separately dissolved in 400 ml. water, and the two solutions are then mixed with stirring, the last traces of nitrous fumes being removed by bubbling air through the liquid. After standing for at least 24 hours any sediment is filtered off and the reagent is ready for use. It should be kept in the dark and decanted for use as required.

Method.

A suitable quantity * of the material is ashed in a vitreosil basin at a temperature below a dull red heat. The ash is twice extracted with hot approximately *N* HCl (using in all not more than 100 ml.) and finally washed with hot water. The filtrate and washings are made up to 200 ml. 100 ml.† of this extract are brought to the boil, and

* A quantity such as will give a final weight of 0·1–0·15 g. mixed sulphates.

† If desired, the whole extract may be taken and the amount of material weighed out for ashing adjusted accordingly.

Commonwealth Bureau of Animal Nutrition

5 ml. of 10 % barium chloride solution are added, and the mixture evaporated to about 25 ml., and then, while hot, made alkaline with strong ammonia (sp. gr. 0·880), and finally allowed to cool. When cold, the precipitate is filtered and washed with 2 % ammonia. The filtrate and washings are evaporated *just to dryness* in a 250 ml. beaker. When cold, 30–35 ml. of alcoholic ammonium carbonate are added, and the whole is thoroughly stirred for 5 minutes. The contents, solid and liquid, of the beaker are transferred to a 100 ml. flask and washed in with more alcoholic ammonium carbonate, and after shaking made up to the mark with this reagent. This mixture is allowed to stand for 2 hours, or preferably overnight, and then filtered through a dry filter into a dry flask. 50 ml. of the filtrate are cautiously evaporated to dryness in a weighed vitreosil basin ($3\frac{1}{2}''$). When cold, the residue is treated with 5 ml. concentrated H_2SO_4 (lead free). The excess acid is evaporated off on a sand-bath, and the residue heated on a naked flame until quite dry. At this stage it is advisable to scatter on a little powdered ammonium carbonate, which facilitates the removal of the last traces of H_2SO_4 and hence the drying of the residue. The dish is then heated in a muffle at a bright red heat for 10–15 minutes, cooled and weighed. It is reheated, cooled and weighed again. The increase in weight of the dish gives the weight of mixed sodium and potassium sulphates.

Potassium.—The mixed sulphates, as above, are dissolved in about 20–25 ml. hot water and filtered through a small filter and washed thoroughly with hot water in small portions, collecting filtrate and washings in a $3\frac{1}{2}''$ porcelain basin. (If the weight of mixed sulphates is 0·2 g. or more, make the filtrate and washings up to 100 ml. and take an aliquot equal to 0·1–0·15 g. of mixed sulphates.) Two or three drops of 10 % NaOH are added to ensure that the solution is just alkaline and then 3–4 drops of glacial acetic acid to make it just acid. One drop of phenolphthalein is added as indicator. The whole is then evaporated on a water-bath to about 5 ml., and 1 ml. glacial acetic acid and 10 ml. sodium cobaltinitrite reagent are added and the evaporation is continued until the residue sets to a fairly stiff paste when cold. When cold, the residue is worked up with 50 ml. of 10 % acetic acid, and when completely disintegrated is left to stand overnight. Next morning the precipitate is filtered on a Gooch crucible with an asbestos pad and washed twice with 50 ml. of 10 % acetic acid and then twice with 50 ml. of water. While the filtration is proceeding about 300 ml. water are brought to the boil in a 600 ml. beaker. When

filtration is complete 35 ml. $N/5$ $KMnO_4$ are run into the boiling water, the burner is removed, and the pad, precipitate and crucible are dropped in. Immediately 25 ml. of 20 % H_2SO_4 are added, and the heating is continued with stirring until the solution is just boiling and all signs of the yellow precipitate have disappeared. If during this process the colour of the permanganate shows signs of disappearing, a further measured volume must at once be added. When the cobaltinitrite precipitate is all decomposed $N/5$ oxalic acid is run in from a burette until the permanganate colour has disappeared and, with slight heating over the burner, any brown manganous hydroxide formed has re-dissolved. The excess oxalic acid is then titrated back with $N/5$ $KMnO_4$.

A blank estimation should be performed by preparing an asbestos pad and dropping it into the boiling water containing 2·5 ml. of $N/5$ $KMnO_4$ and then continuing as above. This blank should not exceed about 0·2 ml.

The total $KMnO_4$ used — (oxalic acid used + blank) gives the number of ml. of permanganate equal to the potassium in the mixed sulphates.

1 ml. $N/5$ $KMnO_4$ ≡ 0·001422 g. K.

(If by any chance the volume of $KMnO_4$ required materially exceeds 35 ml. it may be necessary to repeat the whole estimation, adjusting one's quantities accordingly.)

Sodium.—The mixed sulphates and the K are expressed as percentages of the original material. The percentage of K is converted into percentage of K_2SO_4, and this is subtracted from the percentage of mixed sulphates, giving the percentage of Na_2SO_4 which can then be converted into percentage of Na.

Notes from Oversea Workers.

Husband (S. Rhodesia): For the cobaltinitrite reagent, recommends keeping the cobalt acetate and sodium nitrite solutions in separate opaque bottles and only mixing the required quantity on the day before use. He finds that the cobaltinitrite decomposes in his laboratory, if kept for more than a week or two.

Robinson (Canada): Prefers to weigh the Na and K as mixed chlorides, and to estimate the K by the

platinum chloride method (Lindo-Gladding, *Methods of Analyses, A.O.A.C.*, Washington, 1935, p. 126).

Malan (S. Africa) : Estimates Na direct by the zinc-uranyl acetate method (either gravimetric or colorimetric). The method is only approximate when very small quantities of Na are present.

Aston (New Zealand) : Considers the method of Piper (*J. Soc. Chem. Indust.*, 1934, **53,** 392T) more accurate for estimating K volumetrically as the cobaltinitrite. Estimates Na gravimetrically as the triple sodium magnesium uranyl acetate salt (Piper, *J. Agric. Sci.*, 1932, **22,** 677), using Kahane's reagent.

Sen ; Carberry (India) : Weigh Na and K as the mixed chlorides and then determine K gravimetrically as its perchlorate (*cf. Methods of Analyses, A.O.A.C.*, Washington, 1935, p. 126).

Aykroyd (India) : Precipitates the K directly from the acid ash extract as its perchlorate and weighs as such.

ESTIMATION OF IRON IN FOODSTUFFS AND OTHER MATERIALS.

References.

McFarlane, W. D. *Biochem. J.*, 1932, **26,** 1034.

Fullerton, H. W., Lyall, A. and Davidson, L. S. P., *Quart. J. Med.*, 1933, **2,** 561.

Reagents.

Hydrochloric acid : 6 *N*.

Nitric acid : Concentrated (free from oxides of nitrogen).

Amyl alcohol : (Analar).

Standard iron solution : 3·514 g. Ferrous ammonium sulphate (Analar) are dissolved in a minimum quantity of distilled water. To this 100 ml. of 10 % H_2SO_4 are added and mixed. Then 17 ml. pure concentrated HNO_3 are added to convert the iron to the ferric salt. The mixture is allowed to stand overnight and then made up to one litre with water. This solution keeps indefinitely without the formation of any precipitate
(1 ml. ≡ 0·5 mg. Fe).

Diluted standard : For actual matching 25 ml. of the standard iron solution are diluted to 250 ml. 0·2 ml. of this diluted solution contains 0·01 mg. Fe.

Potassium thiocyanate : 20 % solution.

The purest obtainable reagents must be used and must be tested for the presence of iron. Water *redistilled from glass* must be used throughout.

Method.

About 7 g. (amount varies with class of material being analysed) of material are ashed in a vitreosil basin at a low temperature, cooled, moistened with about 1 ml. HNO_3, dried on a hot plate, and again ashed. This is repeated. The ash is then cooled, moistened with HCl, dried on a hot plate, and again ashed in the low temperature muffle. This also is repeated. The residual ash is now taken up with about 14 ml. of 6 *N* HCl and 50 ml. water and left on the hot plate for at least one hour. It is then cooled, diluted and washed into a 100 ml. flask and made up to the mark. This solution is used for the estimation of iron and of copper.

For iron an aliquot * (usually 1 ml.) is taken in a 50 ml. flask, diluted, one drop of concentrated HNO_3 is added and the solution is allowed to stand for half an hour. 10 ml. of amyl alcohol, carefully measured, and 5 ml. of 20 % potassium thiocyanate are then added. The solution is shaken, made to volume and the amyl alcohol layer pipetted into the colorimeter cup for comparison. If necessary, the amyl alcohol may be centrifuged to clarify prior to matching.

* It is advisable to adjust the amount of this aliquot so that the readings on the two sides of the colorimeter are approximately equal.

As standard for comparison, 0·2 ml. of the diluted standard iron solution is pipetted into a 50 ml. flask, diluted, one drop of concentrated HNO_3 and 0·1 ml. of 6 *N* HCl added. After half an hour, 10 ml. of amyl alcohol and 5 ml. of 20 % potassium thiocyanate are added, the solution shaken and made up to the mark. The amyl alcohol layer is pipetted off, and used for the colour comparison against the unknown.

ESTIMATION OF COPPER IN FOODSTUFFS AND OTHER MATERIALS.

References.

McFarlane, W. D., *Biochem. J.*, 1932, **26,** 1022.
Elvehjem, C. A. and Lindow, C. W., *J. Biol. Chem.*, 1929, **81,** 435.

Reagents.

See iron method for some reagents, (saturated solution) also :

Sodium pyrophosphate :	
Ammonia :	concentrated (sp. gr. 0·880).
Sodium hydroxide :	40 %.
Sodium diethyldithiocarbamate :	2 % aqueous solution. This keeps fairly well when stored in a dark coloured bottle. A precipitate forms in a few weeks. After filtering, the reagent may still be used.
Standard copper solution :	0·3928 g. pure copper sulphate ($CuSO_4$, $5H_2O$) is dissolved in water, a small amount of dilute H_2SO_4 being added to prevent the formation of basic copper sulphate by hydrolysis. The solution is diluted to one litre (1 ml. ≡ 0·1 mg. Cu). In most cases 25 ml. of this solution diluted to 250 ml. (*i.e.* giving 0·01 mg. Cu per ml.) is a suitable standard, but a

series of standards should be prepared and the one nearest the unknown used in the colour comparison.

As in the iron estimation, water *redistilled from glass* must be used throughout.

All reagents must be tested for freedom from copper.

The vitreosil basins to be used for ashing must be cleaned. To each dish add an alcoholic solution of sodium acetate containing 1 g. of the salt, evaporate to dryness and ignite. Then let the dishes stand for several days in HCl (1 : 1) and keep them in this acid when not in use.

Method.

To an aliquot of the ash extract (see method for iron) in a 25 ml. flask, add 1–5 ml. of saturated sodium pyrophosphate, and concentrated ammonia from a burette until the solution is just alkaline to litmus. Place the flasks in a water-bath at 80° C. for 15 minutes. Cool, dilute and add 1 drop of 40 % NaOH, 5 ml. (carefully measured) of amyl alcohol and 0·5 ml. of the 2 % sodium diethyldithiocarbamate, and make up to 25 ml. Shake the mixture vigorously for about one minute. Leave to stand for half an hour, pipette off the amyl alcohol layer when clear and compare with a standard copper solution treated in the same way. If the amyl alcohol is *pure* the colour is stable for about twelve hours. If, after standing, the amyl alcohol is still cloudy it may be filtered, or alternatively transferred to a small test tube having a pellet of filter paper at the bottom, and centrifuged.

For the colour comparison, a series of standards are prepared by taking 0·5, 0·6, . . . 1·0 ml. of the diluted copper sulphate solution in 25 ml. flasks, adding 0·2 ml. HCl to each, and then the same amount of pyrophosphate as used in the unknown, and continuing the method as above.

Note from Oversea Worker.

Aston (New Zealand). Prefers the method of Tompsett (*Biochem. J.*, 1934, **28,** 1536 ; 1935, **29,** 480).

ESTIMATION OF MANGANESE IN FOODSTUFFS AND OTHER MATERIALS.

Reference.

Richards, M. B., *Analyst*, 1930, **55**, 554.

Reagents.

Stock solution of manganese sulphate: Pure $KMnO_4$ (0·144 g.) is dissolved in about 100 ml. of water and reduced by SO_2. Instead of passing in the gas, it is much simpler to generate the gas in the solution itself by means of a little H_2SO_4 and sodium sulphite or bisulphite. The solution is warmed until the reaction takes place, and finally the excess of SO_2 is boiled off. The solution is then diluted to 1 litre (1 ml. ≡ 0·05 mg. Mn).

Standard solution for comparison: Oxidise 20 ml. of the stock solution with periodate in the usual way and make up to 1 litre with 5 % H_2SO_4 previously boiled with periodate (1 ml. ≡ 0·001 mg. Mn).

Both these solutions keep for 3 months in the dark in stoppered flasks.

Method.

A suitable quantity of the material is ashed as completely as possible in a vitreosil basin at a *low* red heat. The residue is treated with a little concentrated HCl and evaporated to dryness. A few ml. H_2SO_4 (33 % by vol.) and 3–4 drops of concentrated HNO_3 are added and the whole evaporated carefully to dryness in a water-bath, finishing on a sand-bath with final gentle ignition over a bunsen flame. To the residue are added 2–2·5 ml. of 33 % H_2SO_4 and a little water and the mixture is evaporated to white fuming stage to remove all traces of chlorides.

After cooling, the residue is diluted with water and filtered into a small flask (50 ml.) for oxidation. To the solution are added one or two small pieces of pumice stone, previously purified by boiling with 5 % H_2SO_4 and a little sodium periodate. The solution is evaporated

down to about 10 ml. when the concentration of the H_2SO_4 will be 5–6 %. To this is added 0·3 g. of sodium periodate (or potassium periodate), a loosely fitting pear-shaped glass stopper being placed in the neck of the flask, and the mixture heated to boiling and immersed in boiling water for 30 minutes. Wooden test tube holders serve to hold the flasks in position in the bath.

If the whole of the solution is required for the colour comparison, it is cooled and transferred for the determinations to the colorimetric tube described below. If the solution has sufficient colour to require dilution, either (*a*) it is diluted to the appropriate volume with 5 % H_2SO_4 which has been boiled with a little periodate, or (*b*) water is added till it has nearly the required dilution and the whole is heated for another 15 minutes in the bath. The solution is transferred to a calibrated flask, made up to volume, and the manganese estimated by comparing with the standard solution.

Colorimetric tube.—The tubes used are 10 ml. calibrated cylinders of uniform bore, with a total capacity of 12–13 ml. and length of 12–13 cm., graduated in tenths of a ml., and made without the usual flange at the bottom, so that the tubes can be held closely together for the colour comparison. These tubes are most suitable for very dilute solutions. With a standard which contains 0·001 mg. Mn per ml., 7–10 ml. give a very convenient depth of tint for matching, but a detectable pink colour can be obtained with as little as 1 ml. and differences of 0·2 ml. give distinctly recognisable differences in tint. If preferred, a colorimeter may be used when the solution is sufficiently strong, but in many cases the amount of manganese in organic materials is so small that it would require an excessive amount of material to give sufficient depth of colour for the colorimeter.

Conditions where modifications are necessary:

For substances containing much calcium, *e.g.* milk.

For substances containing much iron and very little manganese, *e.g.* blood.

(*a*) *Method for milk.*—100 ml. milk in a vitreosil basin are evaporated on the water-bath, with protection from contamination by particles of dust, etc. After ashing completely, and taking down to dryness with HCl, the ash is moistened with a little water, and broken up as finely as possible. 25 ml. of boiling H_2SO_4 (33 % by volume) are added, with careful stirring. After thorough cooling (overnight, if convenient), filter into another vitreosil basin, washing 5–6 times with 5 ml. of the acid. [Test filter paper first for resistance to this acid. If paper will

not stand up to it, use 20–25 % acid, but not more dilute than this.] Evaporate the filtrate to dryness and proceed as usual, except that it may be necessary, when filtering at the final stage before oxidation, to re-filter several times before washing, in order to get an absolutely clear solution.

(*b*) *Method for substances containing much iron and little manganese.*— If the amount of manganese present is minute, and the ratio Fe : Mn is high, *e.g.* in blood and spleen, proceed thus: after evaporation to dryness with H_2SO_4 and ignition over a bunsen burner, instead of adding 2–2·5 ml. of 33 % H_2SO_4, use a mixture of 1–2 ml. of syrupy phosphoric acid and 0·5 ml. H_2SO_4. The phosphoric acid is used to reduce the yellow tint of the iron salt at the final matching stage. Further procedure is as usual. With no other animal tissues have we found this modification necessary. In the case of liver, the high amount of iron does not interfere, on account of the dilution necessitated by the high manganese content of liver.

Note from Oversea Worker.

Aykroyd (India). Uses the method of Newcomb and Sankaran (*Indian J. Med. Res.*, 1929, **16,** 788).

ESTIMATION OF COBALT IN FOODSTUFFS.

Reference.

Stare, F. J. and Elvehjem, C. A., *J. Biol. Chem.*, 1933, **99,** 473.

Reagents.

Standard cobalt solution :	0·2017 g. of pure cobalt chloride ($CoCl_2$, $6H_2O$) in 500 ml. This solution is such that 1 ml. ≡ 0·1 mg. Co.
Indicator solution :	1 g. nitroso - R - salt (sodium 2 : 3 : 6 - β - naphtholdisulphonate) made up to 100 ml. with water.
Sodium acetate :	(Analar).
Potassium hydroxide :	Saturated aqueous solution.

Method.

A suitable amount of the material (preferably containing between 0·05 and 0·5 mg. Co) is thoroughly ashed at a dull red heat. The ash is extracted with a little 50 % HCl, the extract being collected in a small pyrex beaker. To this solution, whose volume should not be more than 20–25 ml., are added 2 drops of phenolphthalein, 2 ml. of indicator reagent, and about 2 g. sodium acetate. The mixture is warmed to about 70° C. and thoroughly stirred. Then with continuous stirring the saturated KOH solution is added slowly, drop by drop, until the reaction is just alkaline. The contents of the beaker are then heated to boiling and concentrated HNO_3 added drop by drop until there is a distinct excess of acid; the boiling is continued for 1 to 2 minutes. A permanent change in colour towards red indicates the presence of cobalt. The colours produced by iron, nickel and other metals are destroyed by oxidation with the HNO_3. The solution is allowed to cool, made up to 50 ml. with water and matched in a colorimeter against a standard cobalt solution treated in the same way. It is better to prepare a series of standards and match against the nearest one.

Note from Oversea Worker.

Aston (New Zealand). Recommends this method, but prefers to use wet ashing as follows: a suitable quantity of the material is digested in a pyrex Kjeldahl flask with a mixture of H_2SO_4 (Analar) and redistilled HNO_3 until a colourless liquid is finally obtained when all the HNO_3 is expelled. The liquid is transferred to a vitreosil basin and evaporated just to dryness on a hot plate. The residue is taken up with 50 % HCl and the estimation completed as above. Aston finds that for most purposes the amount of R-salt used in each estimation can be considerably reduced below the amount recommended by Stare and Elvehjem. Ammonium salts interfere with the development of the colour and some oxidising agents tend to destroy the colour.

ESTIMATION OF ZINC IN FOODSTUFFS AND OTHER MATERIALS.

Reference.

Hibbard, P. L., *Indust. Eng. Chem., Anal. Ed.*, 1934, **6**, 423.

Apparatus.

Jena and some other kinds of glass readily give up zinc to dilute acid. Pyrex and Kavalier glassware seem satisfactory. Ordinary glass bottles and good porcelain appear to be free from soluble zinc.

Method for Plant Material.

Ash 2 g. of plant material in a porcelain basin in a muffle at a low red heat in order to avoid fusion of the ash. Extract the soluble material with about 3 ml. of 3 *N* HCl and 10 ml. water. After half the liquid has evaporated on the steam-bath, add 10–11 ml. of a 10 % solution of oxalic acid. When further evaporation has reduced the total volume to about 20 ml. remove from the heat and, if copper is present, pass in H_2S for a few minutes and let stand till cold, best overnight. Filter out the insoluble residue and the cupric sulphide, together with the oxalates of calcium and lead, and wash with a little 5 % oxalic acid. To the clear filtrate add 10 ml. of 10 % oxalic acid and 1 ml. of 4 % potassium ferrocyanide and mix. A white cloud indicates the presence of zinc. If no cloud appears, appreciable amounts of zinc are absent. After 10 to 15 minutes filter out the zinc ferrocyanide. Addition of 0·02 g. of powdered talc aids in obtaining a clear filtrate. It may be necessary to return the filtrate to the filter once or twice in order to retain all the precipitate. Wash the filter twice with a 5 % solution of oxalic acid, decompose the precipitate on the filter paper with 5–10 ml. of *N* sodium sulphide, and wash three times with some of the same solution diluted with three volumes of water. Lastly, wash once with water. If zinc was present in the solution, zinc sulphide now remains on the filter. Dissolve the zinc off with *N* HCl, returning the filtrate to the filter until it comes clear. In the clear filtrate the zinc is again precipitated by potassium ferrocyanide and estimated nephelometrically.

If desired, centrifuging may be substituted for filtration in making oxalate separations.

Nephelometric procedure.—The zinc solution dissolved off the filter by *N* HCl, as above, is collected in a 20 ml. flat-bottomed specimen tube suitable for comparisons in the manner in which a Nessler tube is used. To the zinc solution add 2 ml. of 5M NaOH and fill up to the mark with *N* HCl. Add 2 drops of 2 % potassium ferrocyanide, and mix quickly. After standing about 15 minutes in order to develop its maximum turbidity, it is compared with similar tubes containing known amounts of zinc precipitated in the same manner. For comparison, set the tubes on a dead black surface where the light strikes the tube perpendicular to its length. Tubes containing 0·02, 0·04, 0·06, 0·08 and 0·10 mg. Zn will usually be sufficient and permit a better estimation of the amount than if a single tube is used to contain the standard which is adjusted to match the unknown by pouring out part of the mixture. The standards keep for an hour or so. The cloudy suspension of zinc ferrocyanide should be pure white, not coloured blue by iron, and the precipitate should not settle perceptibly in one hour. Iron, copper and manganese must be absent. Not more than 0·10 mg. zinc in 20 ml. of solution can conveniently be measured by this method. Use of a nephelometer to measure the turbidity may promote increased accuracy. Even greater precision is obtainable by the use of a photo-electric cell in making the comparisons.

Method for Animal Material.

50 g. (or less, depending upon the zinc content) of wet material are placed in a 500 ml. Kjeldahl flask and a mixture of equal parts conc. H_2SO_4 and conc. HNO_3 added dropwise ; 1 ml. acid is added for each g. of material used. [The HNO_3 must be redistilled from glass and A.R. H_2SO_4 used. All reagents must be tested for zinc.]

The solution is heated gently at first and then more strongly until it ceases to fume brown. HNO_3 is then added gradually and the heating continued until a colourless or pale yellow solution remains. The solution is transferred to a vitreosil basin and the H_2SO_4 removed on a hot plate at a low temperature. When the H_2SO_4 is driven off the residue is taken up twice with 10 ml. of conc. HCl on the water-bath. The solution is centrifuged and the supernatant liquid made up to 50 ml. in a graduated flask. Of this solution a suitable aliquot containing 0·01 to 0·10 mg. Zn is taken for the zinc estimation, evaporated to dryness in a vitreosil basin on the steam-bath and the soluble material extracted with 3*N* HCl as in the method above for plant material, and the zinc is estimated as described.

Commonwealth Bureau of Animal Nutrition

Note from Oversea Worker.

Aston (New Zealand). Recommends the above method, and finds it satisfactory for the estimation of zinc in animal organs and in the mineral limonite.

ESTIMATION OF PHOSPHORUS IN FOODSTUFFS.

Reference.

Richards, M. B. and Godden, W., *Analyst*, 1924, **49**, 565.

Reagents.

Ammonium molybdate solution : Dissolve 121 g. ammonium molybdate in 355 ml. water and then add 60 ml. of ammonia (sp. gr. 0·880). Mix 489 ml. of concentrated HNO_3 and 1149 ml. water in a large porcelain basin, stir and, while stirring, add the molybdate solution slowly. Leave to stand two days, filter and use.

The HNO_3 and ammonium nitrate must be tested prior to use, as samples have been found to contain traces of P_2O_5.

Method.

From 1–2 g. of the material are weighed out into a vitreosil basin and moistened with 5 ml. of a 20 % solution of calcium acetate. The mixture is dried down on a water-bath and then gently ashed to a white ash. The ash is digested for half an hour with hot $2N$ HNO_3 on a boiling water-bath and the extract is filtered into a 250 ml. graduated flask. Filtrate and washings when cold are made up to the mark. A suitable aliquot portion is pipetted into a 500 ml. round-bottomed flask and diluted to about 200 ml. It is made just alkaline to litmus with concentrated ammonia and then just acid with strong HNO_3. 30 ml. of a 50 % solution of ammonium nitrate are now added, the mixture heated to 70–75° C. and 30 ml. of ammonium molybdate

solution, to which 1·5 ml. of strong HNO_3 have been added, are run in and the mixture well shaken. The precipitate is allowed to settle until the liquid is cold, at least one hour being allowed, but the result is not altered if the mixture is left to stand overnight. It is then filtered off, preferably by suction through a disc of hardened filter paper on a Hirsch funnel, but through an ordinary folded filter paper (Whatman, No. 40, 11 cm.) if suction is not available. The precipitate is washed twice with 10 % HNO_3, 3–4 times with 2 % ammonium nitrate and twice with cold water. The precipitate and filter paper disc are washed back into the precipitation flask with cold water and the precipitate dissolved in a known volume of $N/2$ NaOH, about 1–2 ml. in excess being used. If an 11 cm. filter paper is used it is transferred to a beaker containing about 50 ml. of hot water and the necessary known volume of $N/2$ NaOH. The filter paper is pulped by means of a glass rod and the whole is poured through a Hirsch funnel back into the precipitation flask. The paper pulp, which is retained on the funnel, is washed three or four times with hot water, the washings passing into the precipitation flask. 3–4 drops of phenolphthalein are added as indicator and the solution is diluted to about 300 ml. and boiled for 20 minutes. While still hot it is titrated back with $N/2$ H_2SO_4 and an excess of 1–2 ml. of the acid is run in. The boiling is continued for 20 minutes and the liquid allowed to cool. When cold 3 more drops of phenolphthalein are added and the whole titrated back accurately with the $N/2$ NaOH. The end-point is taken as soon as the first definite pink tint is obtained, although the colour will be found to fade fairly rapidly. The total volume of alkali used less the total volume of acid run in gives the volume of alkali equivalent to the phosphorus in the aliquot of the original material.

1 ml. of $N/2$ NaOH $\equiv$ 0·000596 g. P.

It is desirable that the actual amount of P present for precipitation should not exceed 8–9 mg. or there may be some difficulty in washing the precipitate completely free from HNO_3 and ammonium nitrate.

Notes from Oversea Workers.

Stone (S. Rhodesia). Recommends the use of 1 % sodium sulphate as washing liquid. He dissolves the precipitate in 25 ml. of $N/4$ NaOH, or more if necessary, and for each 25 ml. NaOH

adds 5·0 ml. of 40 % formaldehyde, neutralised to phenolphthalein, and then titrates back in the cold with $N/10$ HCl.

Husband (S. Rhodesia). Recommends ashing with $CaCO_3$ and dissolving the ash in HNO_3. Aliquots can then be used for the chlorine determination as well as for phosphorus.

Aston (New Zealand). Prefers to make the final titration with $N/10$ NaOH.

ESTIMATION OF CHLORINE IN FOODSTUFFS.

Reference.

Husband, A. D. and Godden, W., *Analyst*, 1927, **52**, 72.

Method.

A suitable quantity of the material is weighed out into a vitreosil basin and mixed to a paste with one-quarter of its weight of calcium oxide and a little water. The mixture is dried on a water-bath and then gently ashed at a temperature below a dull red heat. The residue is extracted with hot approximately $2N$ HNO_3 and filtered and washed into a 250 ml. flask. Before making to the mark an excess (exact volume to be measured) of $N/10$ $AgNO_3$ is added and the whole well shaken to cause the silver chloride to clot. The mixture is then made up to the mark and filtered through a dry filter into a dry flask. Two portions of 100 ml. each of the filtrate are then titrated with $N/10$ ammonium thiocyanate using iron alum as indicator.

Note from Oversea Worker.

Carbery (India). Ashes in the presence of Na_2CO_3. To the acid extract adds 1 ml. nitrobenzene for each 0·5 g. Cl, before adding the silver nitrate. The silver chloride need not then be filtered off before titration with the thiocyanate (Caldwell and Moyer, *Indust. Eng. Chem., Anal. Ed.*, 1935, **7**, 38).

ESTIMATION OF IODINE IN FOODSTUFFS AND OTHER MATERIALS.

Reference.

The method now in use at the Rowett Institute is that described by C. O. Harvey in *Medical Research Council, Special Report Series* No. 201, entitled " The determination of iodine in biological substances ". The following is a brief account of Harvey's method, but workers are strongly advised to consult the original before using the method, so as to familiarise themselves with all the necessary precautions.

Reagents.

Distilled water : This must be freed from iodine by distillation after rendering the water strongly alkaline by the addition of KOH.

Absolute alcohol : This reagent must be purified so as to leave no organic residue. It is gently boiled under a reflux with 1 % of solid KOH for 20 minutes and then distilled rapidly through an all-glass apparatus, with ground glass joints. The first 10–20 % of the distillate is rejected and the distillation is stopped when only 10–20 % of the original volume remains in the distillation flask. The alcohol is kept in a glass stoppered bottle in a dark cupboard.

Potassium carbonate, iodine free : 500 g. of the carbonate are dissolved in sufficient hot water (about 300 ml.) to produce a complete solution and a solution of 0·25 g. hydrazine sulphate in a little water is added. The solution is heated in a large porcelain dish over a low bunsen flame with frequent stirring, evaporation being allowed to proceed until the mixture, on cooling, forms a stiff pasty mass. The residue is dissolved in sufficient water to make a saturated solution and the latter is repeatedly extracted by shaking, in a separating funnel, with 90–95 % alcohol.

Potassium hydroxide : 10 *N* aqueous solution of iodine-free KOH. 125 g. of KOH are dissolved in sufficient water to make a saturated solution, and after the addition of 0·25 g. hydrazine sulphate, the solution is boiled gently in a flask for about 30 minutes to reduce iodate. When cold the solution is transferred to a separating funnel and extracted by shaking with an equal volume of iodine-free acetone, distilled from potash. The acetone layer is rejected and the extraction is repeated a further nine times with acetone. The aqueous KOH is then boiled gently to remove traces of acetone, cooled, adjusted to 10 *N* and, if necessary, filtered through a Whatman No. 54 filter paper.

Sucrose : 20 % aqueous solution of iodine-free sucrose.

Sulphuric acid : *N*/2.

Bromine water : Liquid bromine, contained in an amber bottle, is shaken with distilled water, the aqueous layer being poured off and rejected, and the extraction with water repeated several times. A further quantity of water is saturated with the washed bromine. This is prepared fresh every day or, if protected from the action of daylight, will remain suitable for use for several days.

Potassium iodide : 2 % aqueous solution. This is preferably prepared fresh each day as required.

Soluble arrowroot starch : 0·5 % solution. 50 g. genuine arrowroot starch are treated at laboratory temperature with 250 ml. of 2*N* HCl, and the mixture allowed to stand, with occasional stirring, for seven days. The acid is then decanted from the starch and the latter is thoroughly washed by decantation, until free from acid. It is finally spread out on filter paper and carefully dried. The solution, *which must be freshly made*, is prepared by rubbing up the starch with a little water and pouring the

mixture into boiling water and the mixture boiled for a total period not exceeding two minutes.

Sodium thiosulphate : A standard $N/10$ solution is prepared with water freed from dissolved gases by boiling and containing 1 % by volume of pure amyl alcohol. The thiosulphate may be standardised against standard potassium bi-iodate. If kept in a dark cupboard the solution is quite stable, but should be standardised at intervals. It is diluted to $N/500$ as required for the actual iodine titrations.

Glass beads : Washed, dried in the oven at 150° C. and ignited in the muffle at 500° C. and kept in a stoppered bottle.

Potassium nitrate : 0·8 % aqueous solution.

Sodium azide : 1·0 % aqueous solution.

Method.

Milk : 40–50 ml. of the milk are treated in a 350 ml. flat-bottomed flask with 4 ml. 10 *N* KOH, the flask being covered with a small beaker and left to stand for 5 or 6 days with occasional shaking until saponification is complete. The solution is homogenised by heating on a water-bath and then transferred completely to a flat-bottomed nickel dish 10 cm. diameter and 2 cm. deep, where it is evaporated on a water-bath. When the contents are nearly dry the dish is transferred to an oven at 150–160° C. and kept there for one hour. It is then placed on an electrically heated sand-bath, at 400–420° C. for two hours. If the mass swells up it is pierced with a finely pointed glass rod. Finally the dish is heated in an electric muffle furnace at 480–500° C. for one hour, attention again being paid to any swelling. The dish is examined periodically and withdrawn for this purpose and also to check any tendency for the contents to take fire or burn with incandescence.

When the dish is cold the material is moistened with about 20 ml. water, the carbon is thoroughly broken up with a glass rod, and the mixture digested with stirring on a water-bath for a few minutes. The hot solution is decanted through a previously washed 9 cm.

Commonwealth Bureau of Animal Nutrition

Whatman No. 30 filter paper into a 100 ml. beaker, the extraction and washing being continued until the filtrate has a volume of about 40 ml. The filtrate at this stage should only be slightly coloured. The filter paper is put back into the nickel dish and the contents of the dish are moistened with water and 0·1 ml. 10 *N* KOH added. After stirring, the rod is removed, the mixture is evaporated to dryness on the water-bath, dried for 15 minutes at 150–160° C. and finally ignited for one hour in the muffle at 480–500° C. The residue is extracted with hot water as before and the combined filtrates are evaporated to dryness in the original nickel dish. The residue is dried in the oven at 150–160° C. for ½ to 1 hour, and ignited in the muffle furnace at 480–500° C. for 5 minutes. The residue is taken up in a little water, the solution being again evaporated to dryness on the water-bath and after drying at 150–160° C. the dish is again heated in the muffle for 5 minutes.

The alkaline mass should now contain little carbon and is dissolved in about 25 ml. water. The solution is digested on the water-bath and filtered through a 7 cm. Whatman No. 42 filter paper, previously well washed with hot water, washing two or three times with about 3 ml. *N*/10 K_2CO_3. The filtrate and washings are evaporated to dryness in an ignited 3½″ porcelain basin, the residue being dried for 30 minutes in the oven at 150–160° C. and finally ignited for 5 minutes in the muffle furnace at 480–500° C.

The alkaline mass which should now be practically free from carbon, is rubbed up to a smooth cream with sufficient water to produce an almost complete solution. About 2 ml. water suffice if 4 ml. 10 *N* KOH were originally used. It is better to use too much rather than too little water. The mass is left to stand for some time, after being moistened with the water, before rubbing it up to a cream. 5 ml. alcohol are added and the mixture well rubbed with a small glass pestle. A further 5 ml. alcohol are added, the mixture again rubbed up, and finally a third 5 ml. alcohol added. The rubbing is continued until the K_2CO_3 passes into a thin pasty condition, when the alcoholic layer is carefully decanted into an ignited platinum dish 8 cm. in diameter. The residue is extracted with a further 5 ml. alcohol, which is decanted off and a third extraction is carried out with another 5 ml. alcohol. The pasty condition may be maintained by the addition, if necessary, of small quantities of water.

The combined alcoholic extracts are treated with 0·15 ml. *N*/1 solution of K_2CO_3 and rapidly evaporated under cover in a current of nitrogen. The dish is dried at 150–160° C. in the oven for 5 minutes,

and the residue is treated with 0·5 ml. 0·8 % potassium nitrate and 1–2 ml. water. The solution is evaporated to dryness on the water-bath, the residue dried in the oven at 150–160° C. for 5 minutes, and ignited in the muffle at 480–500° C. for *two minutes only*.

During all the process after the extraction with alcohol, care must be taken to avoid contamination with dust.

The residue in the platinum dish is dissolved in 0·5 ml. of 1 % solution of sodium azide and 2 ml. water, and the solution carefully transferred to the small (approx. 10 ml.), wide-necked, round-bottomed, titration flask, the transference being completed by thoroughly washing the dish twice with 1 ml. water. The alkaline liquid must on no account come in contact with the neck of the flask. Make the solution *just* acid with $N/2$ H_2SO_4, using a finely pointed glass rod to take a small drop for " spotting " on a methyl orange paper, and then add a further 0·05 ml. of the $N/2$ acid and 0·2 ml. of saturated bromine water. Finally, add a glass bead and at once boil off the bromine on an asbestos heating board with a circular hole in which the flask rests. Heat by means of a micro-bunsen burner, and so adjust the flame that the liquid is boiled down to 2 ml. in $2\frac{1}{2}$ to 3 minutes. Cool rapidly in a stream of cold water, add 0·1 ml. of $N/2$ H_2SO_4, 0·1 ml. starch solution, and 0·1 ml. KI solution and titrate the liberated iodine with $N/500$ sodium thiosulphate, using a Rehburg burette.

Frequent blank determinations must be carried out to correct for traces of iodine that may be present in the reagents, filter papers, etc., and to ensure that iodine is not being introduced from any source. For these blanks use 5 ml. of the iodine free sucrose solution, and go through the complete process.

Vegetable material: 3–5 g. of the dry ground material are digested on a water-bath for 2–3 hours in a 350 ml. flat-bottomed flask with 3·0 ml. of 10 N KOH solution. The contents are then transferred to a nickel dish and the estimation carried out as above.

Blood: 10 ml. blood are digested with 20 ml. water and 1·5 ml. 10 N KOH in a flask on a water-bath for 2–3 hours with occasional shaking and then the determination is completed by the standard method. Care must be taken in the ashing process as the dry mass is liable to swell considerably.

Notes from Oversea Workers.

Malan (S. Africa). Uses the method described by Blom (*Onderstepoort J. Vet. Sci.*, 1934, **2,** 123). Accuracy ± 20 %.

Aykroyd (India). Uses the method of Patnaik (*Indian J. Med. Res.*, 1933, **21,** 237; 1934, **22,** 249).

ESTIMATION OF TOTAL SULPHUR IN FODDER AND EXCRETA.

Reference.

Marston, H. R. (Australia), Private communication.

Method.

Fodder is sampled and ground in a Wiley mill until it all passes a 0·5 mm. mesh. Sheep faeces are similarly treated. In each case the ground material is spread out on glazed paper to come into equilibrium with the laboratory air. A measured volume of urine is evaporated in a serum drier at 40° C. to the consistency of thick treacle and samples for the determination are drawn from this.

The oxidation is carried out in an Emerson bomb. Approximately 2 g. of the dry material (fodder or faeces) are compressed into a hard tablet around an iron fuse wire by means of a die; or about 2 g. of evaporated urine are transferred to a bomb capsule. The material is then weighed on the capsule, upon which it is later ignited in the bomb, allowance being made for the iron fuse wire, which is previously weighed. The capsule and its contents are then set up in the bomb, and the fuse wire fixed in position. *The lead washer of the bomb is then completely covered with a pure tin gasket,* and the bomb assembled and tightly screwed down. Pure oxygen is then let in to a pressure of twenty atmospheres, the valves closed and the bomb immersed in water to detect any leak of oxygen. The bomb is then fired electrically. Complete combustion results, the silica and basic elements being fused into clear glass beads and the iron wire completely transformed to Fe_2O_3. A large proportion of the nitrogen in the sample is burned to HNO_3 and all the sulphur is transformed to SO_3. When the bomb is

cool the valve is opened to relieve the pressure. The contents of the bomb are then completely washed out into a pyrex beaker, acidified with excess HCl and taken twice to dryness at 100° C. to free from HNO_3. The residue is taken up with HCl, filtered off and washed, and the filtrate adjusted in acidity to 0·02 *N* in a final volume of about 250 ml.

The precipitation is carried out as follows : the perfectly clear and faintly acid solution is brought to incipient boiling on an electric hot plate, excess of hot barium chloride solution is added, and the beaker covered with a clock glass. It is allowed to remain at approximately 100° C. for a further two hours, when the current is turned off from the hot plate and the temperature allowed to fall slowly overnight. Next day the major quantity of the supernatant liquid is sucked off through a bent capillary and the precipitate quantitatively transferred to a Whatman No. 44 filter paper, where it is thoroughly washed. The paper and precipitate are then dried in a platinum crucible and ignited in an electric muffle. The residue is carefully " fumed off " with a drop of pure HNO_3 and re-ignited at a temperature of about 500° C. The lid is then placed on the crucible, which is allowed to cool in the air of the balance room and weighed, the empty crucible having been previously similarly treated and weighed. This procedure is considered preferable to cooling in a desiccator and weighing in the " dry " atmosphere of the balance case, in which desiccating agents are exposed, when only small differences are being sought.

Meticulous care is necessary in all the manipulations and checks must be carried out for possible presence of sulphur in the reagents used.

This method was found to be preferable, and much more reliable, for the materials examined than either the sodium peroxide fusion or the Benedict-Denis method.

II. ANALYSIS OF BLOOD.

GENERAL REMARKS.

Drawing samples.—In a series of analyses over a period of weeks, it is desirable that animals should always be bled at the same hour of the day and have been disturbed as little as possible beforehand. For ordinary purposes we find the jugular vein the most convenient of access, the blood being drawn with partial stasis through an oiled needle and the first few ml. rejected.

Preparation of samples for analysis :

(1) Serum : We find that the placing of a small plug of glass wool in the top of the blood sample materially expedites clotting and ensures complete precipitation of the fibrin.

(2) Plasma : Centrifuge first in a round-bottomed centrifuge tube. Pipette off the top layer and centrifuge again for 5 minutes in a conical centrifuge tube to ensure complete compaction and removal of white cells.

(3) Oxalated blood : Do not use more than 1 drop of 20 % neutral potassium oxalate as anticoagulant for each 5 ml. of blood to be collected. This drop should be spread round the tube and dried in an oven and the oxalate broken up with a fine rod.

(4) For the estimation of blood inorganic phosphorus the 2 ml. of oxalated blood should be pipetted into the trichloracetic acid immediately after it is drawn (*i.e.* in the byre) and the analysis proceeded with as quickly as possible thereafter.

ESTIMATION OF SERUM CALCIUM.

Reference.

Clark, E. P. and Collip, J. B., *J. Biol. Chem.*, 1935, **63,** 461.

Reagents.

Sulphuric acid :	Approx. *N*.
Ammonium oxalate :	Half-saturated solution.
Sodium oxalate :	*N*/100 containing 1 ml. H_2SO_4 per litre.
Potassium permanganate :	*N*/10. This is diluted to *N*/100 when required.
Dilute ammonia :	20 ml. concentrated ammonia (sp. gr. 0·880) diluted to 1 litre with water.

Method.

Into a 5 ml. conical centrifuge tube measure 2 ml. of the half-saturated ammonium oxalate solution (accurately by micro-burette if serum Mg is to be subsequently estimated), and add 2 ml. serum. Twirl the tube between the fingers, mix the two liquids and then leave to stand for 4 hours. Centrifuge. Pour off the supernatant fluid into a dry test tube and put aside for serum Mg estimation. Invert the centrifuge tube in a rack to drain for 5 minutes. Dry the inside and mouth of the tube with filter paper and wash the precipitate and walls of the tube with 2–3 ml. dilute ammonia. Centrifuge, drain and dry as before. Then blow 2 ml. of the *N* H_2SO_4 on to the precipitate. Place the tube in a boiling water-bath for 1 minute and finally titrate with *N*/100 $KMnO_4$.

No. of ml. of *N*/100 $KMnO_4 \times 10 \equiv$ mg. Ca per 100 ml. serum. Periodically a blank should be run on the reagents and the amount, if any, should be subtracted.

Notes from Oversea Workers.

Stone (S. Rhodesia). Thinks it may take more than one minute to dissolve the calcium oxalate in the H_2SO_4. (This is obviated if the acid is blown on as stated.—*Ed.*)

Baskett (N. Ireland). Uses Tisdall's modification of the method of Kramer and Tisdall (*J. Biol. Chem.*, 1923, **56,** 439). Finds it unsatisfactory with the sera of laying hens. Possible alternative methods in such cases are those of Roe and Kahn (*J. Biol. Chem.*, 1929, **81,** 1) and Roepke and Hughes (*J. Biol. Chem.*, 1935, **108,** 79).

ESTIMATION OF SERUM MAGNESIUM.

References.

Fiske, C. H. and Subbarow, Y., *J. Biol. Chem.*, 1925, **66,** 375.
Holzapfel, C. R., *Onderstepoort J. Vet. Sci.*, 1934, **2,** 115.

Reagents.

Molybdic acid :	To 86 ml. water add 10 ml. molybdate I as in method for inorganic phosphorus.
Standard phosphate solution :	0·0560 g. KH_2PO_4 dissolved in 1 litre of $N/20$ H_2SO_4.
Aminonaphtholsulphonic acid :	As in method for inorganic phosphorus.
Holzapfel's washing reagent :	Mix together 580 ml. 96 % alcohol, 320 ml. water, 100 ml. amyl alcohol and 30 ml. concentrated ammonia (sp. gr. 0·880).
Sulphuric acid :	10 *N*.
Ammonium hydrogen phosphate :	5 % solution ; filter before use.
Ammonia :	Concentrated (sp. gr. 0·880). This must be fresh.

Where directions are not given in full regarding the preparation of reagents these will be found under the method for inorganic phosphorus.

Method.

Into a 12 ml. conical centrifuge tube place 1 ml. concentrated ammonia and then 1 ml. of the 5 % ammonium hydrogen phosphate solution. A precipitate forms, which dissolves on addition of 3 ml. of

the supernatant fluid from the serum Ca estimation. The contents of the tube are mixed by twirling the tube between the fingers, care being taken that no crystals of ammonium hydrogen phosphate remain undissolved, and the tube is then stoppered. The precipitation requires overnight standing to become complete. The precipitate is then compacted by centrifuging for 4 minutes. The supernatant fluid is

TABLE FOR READING MAGNESIUM VALUES. 3 ml. OF STANDARD USED. STANDARD SET AT 15 m.m.

MAGNESIUM VALUES IN mg. PER 100 ml. SERUM.

Unknown m.m.	Mg mg.	Unknown m.m.	Mg mg.	Unknown m.m.	Mg mg.	Unknown m.m.	Mg mg.
10·1	2·98	13·1	2·29	16·1	1·86	19·1	1·57
10·2	2·94	13·2	2·27	16·2	1·85	19·2	1·56
10·3	2·91	13·3	2·26	16·3	1·84	19·3	1·55
10·4	2·88	13·4	2·24	16·4	1·83	19·4	1·55
10·5	2·86	13·5	2·22	16·5	1·82	19·5	1·54
10·6	2·83	13·6	2·20	16·6	1·81	19·6	1·53
10·7	2·80	13·7	2·19	16·7	1·80	19·7	1·52
10·8	2·78	13·8	2·17	16·8	1·79	19·8	1·52
10·9	2·75	13·9	2·16	16·9	1·78	19·9	1·51
11·0	2·73	14·0	2·14	17·0	1·76	20·0	1·50
11·1	2·70	14·1	2·13	17·1	1·75	20·1	1·49
11·2	2·68	14·2	2·11	17·2	1·74	20·2	1·49
11·3	2·65	14·3	2·10	17·3	1·73	20·3	1·48
11·4	2·63	14·4	2·08	17·4	1·72	20·4	1·47
11·5	2·61	14·5	2·07	17·5	1·71	20·5	1·46
11·6	2·59	14·6	2·05	17·6	1·70	20·6	1·46
11·7	2·56	14·7	2·04	17·7	1·69	20·7	1·45
11·8	2·54	14·8	2·03	17·8	1·69	20·8	1·44
11·9	2·52	14·9	2·01	17·9	1·68	20·9	1·44
12·0	2·50	15·0	2·00	18·0	1·67	21·0	1·43
12·1	2·48	15·1	1·99	18·1	1·66	21·1	1·42
12·2	2·46	15·2	1·97	18·2	1·65	21·2	1·42
12·3	2·44	15·3	1·96	18·3	1·64	21·3	1·41
12·4	2·42	15·4	1·95	18·4	1·63	21·4	1·40
12·5	2·40	15·5	1·94	18·5	1·62	21·5	1·40
12·6	2·38	15·6	1·92	18·6	1·61	21·6	1·39
12·7	2·36	15·7	1·91	18·7	1·60	21·7	1·38
12·8	2·34	15·8	1·90	18·8	1·60	21·8	1·38
12·9	2·33	15·9	1·89	18·9	1·59	21·9	1·37
13·0	2·31	16·0	1·88	19·0	1·58	22·0	1·36

poured off, the tube inverted in a rack for 5 minutes to drain and the inside of the tube wiped with filter paper. 5 ml. of the washing reagent are blown on to the mat of magnesium ammonium phosphate to break it up, a portion of the washing reagent being used to wash down the wall of the tube. Three such washings are performed, centrifuging, draining and drying after each, as above. After the final washing the tubes are placed in an oven for one hour to dry. To each tube are added 9·6 ml. of the molybdic acid reagent and 0·4 ml. of the aminonaphtholsulphonic acid. The contents are vigorously shaken and read in the colorimeter after 10 minutes. The standard is made with 3 ml. of the standard phosphate solution, 5·6 ml. water, 1 ml. molybdate I and 0·4 ml. aminonaphtholsulphonic acid.

Calculation :

$$\frac{\text{No. of ml. standard phosphate solution} \times 10}{\text{reading of unknown}} = \text{mg. Mg per 100 ml. serum.}$$

The above expression is for the standard set at 15 m.m.

In cases where, from the bulk of the precipitate, it is obvious that the Mg is low the colour is developed in a final volume of 5 ml. by using half quantities of the molybdic acid reagent and the aminonaphtholsulphonic acid.

Note from Oversea Worker.

Malan (S. Africa). Uses hydroquinone and sodium sulphite as reducing agent. (We have found that aminonaphtholsulphonic acid gives a more rapid colour development.—*Ed.*)

ALTERNATIVE TECHNIQUE FOR ESTIMATION OF SERUM CALCIUM AND MAGNESIUM.

Reference.

Green, H. H. and Allcroft, W. M. (Weybridge), Private communication.

Reagents.

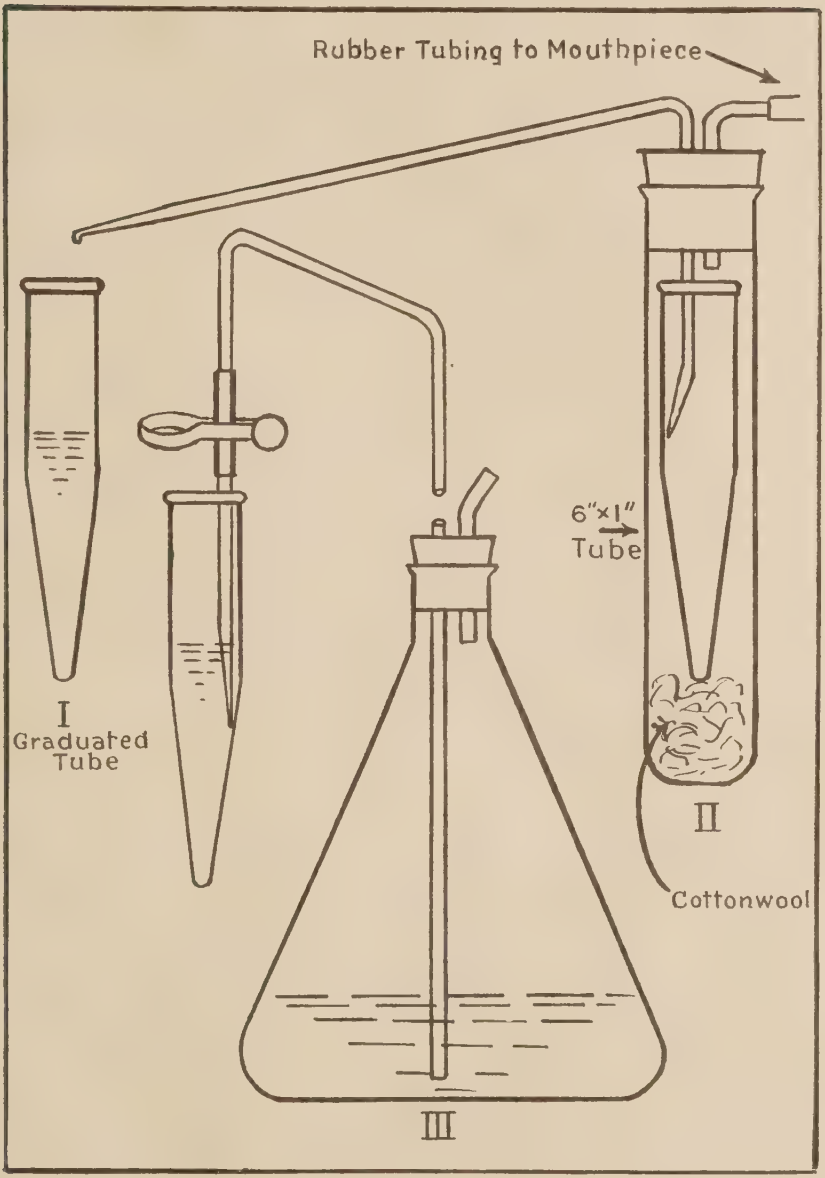
Ammonium oxalate solution : 4 %.

Ammonia : 2 % ; 2 ml. NH_4OH (sp. gr. 0·88) per 100 ml.

Ammonium phosphate solution :	5 %.
Concentrated ammonia :	A.R. (sp. gr. 0·88).
Ammonia :	33 %.
Ammoniated alcohol wash :	5 ml. conc. NH_4OH and 95 ml. 75 % alcohol per 100 ml.
Sulphuric acid :	10 *N*.
Sulphuric acid :	*N*/1.
Potassium permanganate solution :	*N*/10 ; to be diluted 1 in 10 as required.
Sodium molybdate (P-free) solution :	7·5 %.
Molybdic-sulphuric reagent :	100 ml. 7·5 % sodium molybdate, 50 ml. 10 *N* H_2SO_4 and 50 ml. water.
Aminonaphtholsulphonic acid reagent :	In 195 ml. 15 % sodium bisulphite solution which has been freshly filtered after being made up for 2 or 3 days dissolve 0·5 g. 1 : 2 : 4-aminonaphtholsulphonic acid. Add just sufficient 20 % sodium sulphite solution (200 g. in 380 ml. if the salt used is Na_2SO_3, $7H_2O$) to render the final solution clear.
Stock phosphate solution :	0·560 g. KH_2PO_4 per litre. This solution is diluted 1 in 10 for normal use ; 1 ml. of the diluted standard is equivalent to 0·01 mg. magnesium. 4 ml. is the normal standard used.

Method.

The method employed at the Veterinary Laboratory, New Haw, Weybridge, is as follows : place 2 ml. distilled water in a tapered centrifuge tube of the type shown in Fig. I, run into this 2 ml. serum and mix by rotation between the hands, add 1 ml. 4 % ammonium oxalate solution, taking care that none of this solution gets on the upper inside part of the tube. Mix well by rotation between the hands, but do not invert the tube. Leave for 2 hours to allow precipitation of the calcium, and then centrifuge for 5 minutes at 2500 r.p.m.



Scale = ½.

III—Overall height of syphon tube at least 24". Flask, 1-2 litres capacity.

Into a fresh tapered centrifuge tube graduated at 10 ml. run 1 ml. of 5 % ammonium phosphate, taking care that none of the solution gets on to the upper part of the tube, and then, when ready to transfer the supernatant fluid from the calcium oxalate precipitate, run in 1 ml. concentrated ammonia (sp. gr. 0·880) which is fresh and has not been exposed to air. In the hand-siphon, Fig. II, transfer all the supernatant fluid from the calcium oxalate precipitate into the graduated tube, after which all the white precipitate formed when the ammonia was added to the ammonium phosphate will disappear. Cork this tube and mix well by rotation, but not by inverting, and leave overnight for the magnesium ammonium phosphate to settle out.

Returning to the calcium oxalate precipitate: break it up with a small amount of 2 % ammonia from a 5 ml. pipette and use the remainder of the 5 ml. volume completely to wash the upper part of the inside of the centrifuge tube: wipe the lip and inside of the top of the tube with a hard filter paper (Whatman No. 50) on a glass rod. Centrifuge for 5 minutes at 2500 r.p.m. Siphon off the supernatant fluid with the large siphon (Fig. III). Wash again with 5 ml. 2 % ammonia. Centrifuge for 5 minutes at 2500 r.p.m. Siphon off the supernatant fluid. Add 2 ml. N H_2SO_4 to the calcium oxalate precipitate and place the tube in a boiling water-bath, shake gently at intervals till the precipitate has all dissolved and titrate, as rapidly as convenient with accuracy, while still hot, with $N/100$ $KMnO_4$ solution, made fresh by diluting $N/10$ $KMnO_4$ ten times.

No. of ml. $N/100$ $KMnO_4$ $\times$ 10 = mg. Ca per 100 ml. serum.

Next day centrifuge the Mg tube, after removal of the stopper, for 5 minutes at 2500 r.p.m. Siphon off the supernatant fluid and wash the precipitate with 5 ml. 33 % ammonia, observing similar precautions as for the first washing of the calcium oxalate precipitate. Centrifuge again, siphon off the supernatant fluid, wash again with 5 ml. 33 % ammonia. Centrifuge again, siphon off liquid, wash with 5 ml. ammoniated alcohol wash. Centrifuge, siphon off the liquid and dry the tubes in an oven at 100° C.

Colorimetric estimation of the phosphorus present is carried out by means of the Fiske and Subbarow reagents. To the tube add 0·5 ml. 10 N H_2SO_4 and water up to 7 ml. Mix by rotation, add 2 ml. molybdic-sulphuric acid reagent, and mix again, add 0·4 ml. 1 : 2 : 4-amino-naphtholsulphonic acid reagent, make up to 10 ml. and mix by inversion.

Leave for ½ hour and read against a standard P made up at the same time and treated in the same way.

On normal blood 4 ml. of the standard P solution is taken, and the same reagents added as to the unknown, but when the serum Mg is seen to be either high or low, as evidenced by the precipitate of magnesium ammonium phosphate, a graduated series of standards are made and the unknown is read against the standard to which it nearest approximates. For high serum Mg values a stronger standard, 4 or 5 times the standard mentioned, is used and the volume kept within the required limits.

Colorimeter standard cup at 20 m.m. and 4 ml. of standard.

$$\frac{\text{reading of standard}}{\text{reading of unknown}} \times 2 = \text{mg. Mg per 100 ml. serum.}$$

Points to which attention should be paid are :

1. All tubes must be cleaned thoroughly in a cleaning mixture so that they are not greasy or sticky.
2. None of the vital precipitants, ammonium oxalate or phosphate, should be allowed on the upper part of the tube, owing to the risk of not being fully washed off and giving a high result.
3. The process of siphoning off the liquid gives a better chance to see that no precipitate is lost in getting rid of the supernatant fluid, and is to be preferred to a decantation process.

ESTIMATION OF BLOOD INORGANIC PHOSPHORUS.

Reference.

Fiske, C. H. and Subbarow, Y., *J. Biol. Chem.*, 1925, **66,** 375.

Reagents.

Sulphuric acid :	10 *N*; add 450 ml. concentrated H_2SO_4 to 1300 ml. water.
Molybdate I :	Dissolve 25 g. ammonium molybdate in 400 ml. water ; add 500 ml. 10 *N* H_2SO_4 and dilute to 1 litre.

Molybdate II :	Dissolve 25 g. ammonium molybdate in 400 ml. water ; add 300 ml. 10 *N* H_2SO_4 and dilute to 1 litre.
Molybdate II (diluted) :	Mix 25 ml. molybdate II and 90 ml. water. Make fresh daily.
Trichloracetic acid :	13·3 % aqueous solution.
Phosphate standard :	Dissolve 0·3509 g. KH_2PO_4 in water ; add 10 ml. 10 *N* H_2SO_4 and make to 1 litre ; 5 ml. contains 0·4 mg. P.
Aminonaphtholsulphonic acid :	Dissolve 0·5 g. of the acid, 30 g. sodium bisulphite and 6 g. sodium sulphite (cryst.) in water and make up to 250 ml.

Method.

Blow 2 ml. of oxalated whole blood, serum or plasma, into 6 ml. 13·3 % trichloracetic acid in a test tube. Fill the pipette once with water and allow it to empty slowly into the test tube. Shake vigorously, allow to stand for a few minutes and then filter. Proceed with the analysis as quickly as possible, as follows: Pipette, in duplicate, 1·25 ml. of the filtrate into test tubes, add 1·15 ml. molybdate II (diluted) and rotate between the fingers to mix. Add 0·1 ml. aminonaphtholsulphonic acid and invert once to mix. For the standard take 5 ml. of the standard phosphate solution, 70 ml. water and 10 ml. molybdate I in a 100 ml. flask. Mix well, add 4 ml. aminonaphtholsulphonic acid, dilute to the mark, mix well and use in 10 minutes.

Calculation :

$$\frac{80}{\text{reading of unknown}} = \text{mg. P per 100 ml.}$$

The above holds if the 5 ml. standard is set at 20 m.m.

For this method do not use more than 1 drop of 20 % neutral potassium oxalate solution as anticoagulant for each 5 ml. blood. If sodium fluoride is used as anticoagulant add to each 5 ml. blood filtrate 1 ml. 20 % aluminium chloride solution. This depresses the fluoride ion by converting it to aluminium fluoride, which is weakly ionized. Correct the P value found for this dilution.

Note from Oversea Worker.

Malan (S. Africa). Uses the method of Malan and Van der Lingen (17th *Rep. Director Vet. Services and Animal Indust., Union S. Africa*, 1931, 443).

5 ml. STANDARD USED. STANDARD SET AT 20 m.m. (FISKE-SUBBAROW METHOD).

RESULTS IN mg. P PER 100 ml. BLOOD, PLASMA OR SERUM.

Unknown m.m.	P mg.	Unknown m.m.	P mg.	Unknown m.m.	P mg.	Unknown m.m.	P mg.
13·1	6·11	16·1	4·97	19·1	4·19	22·1	3·62
13·2	6·06	16·2	4·94	19·2	4·17	22·2	3·60
13·3	6·01	16·3	4·91	19·3	4·15	22·3	3·59
13·4	5·97	16·4	4·88	19·4	4·12	22·4	3·57
13·5	5·93	16·5	4·85	19·5	4·10	22·5	3·56
13·6	5·88	16·6	4·82	19·6	4·08	22·6	3·54
13·7	5·84	16·7	4·79	19·7	4·06	22·7	3·52
13·8	5·80	16·8	4·76	19·8	4·04	22·8	3·51
13·9	5·76	16·9	4·73	19·9	4·02	22·9	3·49
14·0	5·71	17·0	4·71	20·0	4·00	23·0	3·48
14·1	5·67	17·1	4·68	20·1	3·98	23·1	3·46
14·2	5·63	17·2	4·65	20·2	3·96	23·2	3·45
14·3	5·59	17·3	4·62	20·3	3·94	23·3	3·43
14·4	5·56	17·4	4·60	20·4	3·92	23·4	3·42
14·5	5·52	17·5	4·57	20·5	3·90	23·5	3·40
14·6	5·48	17·6	4·55	20·6	3·88	23·6	3·39
14·7	5·44	17·7	4·52	20·7	3·86	23·7	3·37
14·8	5·40	17·8	4·49	20·8	3·85	23·8	3·36
14·9	5·37	17·9	4·47	20·9	3·83	23·9	3·35
15·0	5·33	18·0	4·44	21·0	3·81	24·0	3·33
15·1	5·30	18·1	4·42	21·1	3·79	24·1	3·32
15·2	5·26	18·2	4·40	21·2	3·77	24·2	3·31
15·3	5·23	18·3	4·37	21·3	3·76	24·3	3·29
15·4	5·19	18·4	4·35	21·4	3·74	24·4	3·28
15·5	5·16	18·5	4·32	21·5	3·72	24·5	3·27
15·6	5·13	18·6	4·30	21·6	3·70	24·6	3·25
15·7	5·10	18·7	4·28	21·7	3·69	24·7	3·24
15·8	5·06	18·8	4·26	21·8	3·67	24·8	3·23
15·9	5·03	18·9	4·23	21·9	3·66	24·9	3·21
16·0	5·00	19·0	4·21	22·0	3·64	25·0	3·20

ESTIMATION OF PLASMA PHOSPHATASE (KAY).

Reference.

Kay, H. D., *J. Biol. Chem.*, 1930, **89,** 235.

Reagents.

Trichloracetic acid :	22 % solution.
Anticoagulant :	1 drop 20 % neutral potassium oxalate solution per 5 ml. blood.
Substrate :	50 ml. 1·5 % sodium β-glycerophosphate and 5 ml. 1·8 % sodium chloride solution. p_H to be adjusted to 7·6 with HCl or NaOH and liquid saturated with chloroform.
Molybdic acid :	Dissolve 25 g. ammonium molybdate in 400 ml. water. Add 75 ml. conc. H_2SO_4 and dilute to 500 ml.
Hydroquinone solution :	0·5 % solution in water with 2–3 drops of conc. H_2SO_4. To be made up fresh each day.
Sodium sulphite :	20 % solution of the crystals. To be made up fresh each day.
Phosphate standard :	Use the Fiske-Subbarow standard phosphate solution (see method for blood inorganic P).

Method.

To 5·5 ml. substrate, in duplicate, add 0·5 ml. plasma. Add to each tube 1 ml. 22 % trichloracetic acid, shake and stopper. Keep in the refrigerator until required for analysis. If a refrigerator is not available analyse immediately. To two tubes containing 5·5 ml. substrate each, add 0·5 ml. plasma, mix, stopper and place in the incubator at 37° C. for 48 hours. To each of these tubes are added a few drops of chloroform to prevent mould growth. At the end of the incubation period add to each, as in each of the blanks above, 1 ml. 22 % trichloracetic acid. Filter. Take the filtrates from the blanks and incubated samples and measure 4 ml. in the case of the blanks and 2 ml. in the case of the incubated samples and place in separate tubes. Make each up to 7 ml. with water, and then add to each 1 ml. molybdic acid, 1 ml. hydroquinone solution and 1 ml. sodium sulphite solution

in that order. Shake and read after 45 minutes. The standards are prepared in the same way by taking different volumes of the phosphate standard, and an amount of 22 % trichloracetic acid equal to that in the filtrate being analysed. Then dilute to 7 ml. and add the other reagents as above.

Calculation :

$$\text{mg. P per 1 ml. plasma} = \frac{\text{Cs} \times \text{Rs} \times 7 \times 2}{\text{Ru} \times \text{Vu}}$$

where

Cs = concentration of standard ;
Rs = reading of standard ;
Ru = reading of unknown ;
Vu = volume of filtrate taken.

The difference between incubated samples and blanks is the phosphate liberated by enzyme activity.

ESTIMATION OF SERUM PHOSPHATASE (BODANSKY).

References.

Bodansky, A., *J. Biol. Chem.*, 1932–33, **99,** 197.
Bodansky, A., *J. Biol. Chem.*, 1933, **101,** 93.

Reagents.

Sulphuric acid : 10 *N*.

Sodium molybdate : 7·5 % solution. 90 g. molybdic acid (highest purity, ammonia and phosphate free) are dissolved in 250 ml. 5*N* NaOH and then diluted to 2 litres. The reagent should be faintly alkaline to phenolphthalein. Decant when required.

Stannous chloride : 60 % solution. 15 g. stannous chloride are dissolved in conç. HCl and made up to 25 ml. with the acid. Do not keep longer than one month. The reagent is always kept in the refrigerator.

Standard phosphate stock solution :	110 mg. KH_2PO_4 are dissolved in water and made up to 250 ml. after adding 1 ml. conc. H_2SO_4 as a preservative. 10 ml. contain 1 mg. P.
Diluted phosphate solution :	10 ml. stock phosphate solution are diluted to 250 ml. and preserved with 1 drop toluene.
Molybdic acid solution :	To 1 vol. of cold 10 *N* H_2SO_4 add rapidly 1 vol. of the 7·5 % sodium molybdate solution, shaking vigorously the whole time. Then add 2 vols. of water with shaking. Prepare fresh daily.
Diluted stannous chloride solution :	Dilute with water 1 ml. of the 60 % solution to 200 ml. Prepare fresh daily.
Buffered substrate :	Dissolve 2·5 g. sodium β-glycerophosphate and 2·12 g. monosodium diethylbarbiturate (known also as barbital-sodium or sodium malourea) in 500 ml. water. This should give a p_H of 8·6, but the reagents obtained in England may give a p_H of 9·2. This is adjusted to p_H 8·6 with dilute H_2SO_4. Make up this reagent as required.
Trichloracetic acid :	10 % solution.
Trichloracetic acid :	5 % solution.

Reagent blank.—Mix together 5 ml. 5 % trichloracetic acid and 4 ml. molybdic acid solution and blow in, from a pipette calibrated for the purpose, 1 ml. diluted stannous chloride solution. The colour developed should be at the most a very faint blue or green.

Method.

Incubated samples.—Into each of two 30 ml. test tubes place 10 ml. buffered substrate. Place in the incubator to raise the temperature to 37° C. and then add to each 1 ml. serum. Mix and return to the incubator and leave for exactly 1 hour. Remove and place in ice water. To each add 9 ml. 10 % trichloracetic acid, shake, allow to stand for a few minutes and then filter.

Blanks.—In each of two test tubes place 9 ml. 5 % trichloracetic acid and add 1 ml. serum. Shake, allow to stand and filter. These

INORGANIC P IN ALIQUOT, AT STATED COLORIMETRIC READINGS. CORRECTED FOR DEVIATION FROM BEER'S LAW. 0·02 mg. STANDARD SET AT 20 m.m.

m.m. m.m.	0·0 mg.	0·1 mg.	0·2 mg.	0·3 mg.	0·4 mg.	0·5 mg.	0·6 mg.	0·7 mg.	0·8 mg.	0·9 mg.
8	0·0560	0·0552	0·0545	0·0538	0·0531	0·0525	0·0518	0·0512	0·0505	0·0499
9	·0493	·0487	·0482	·0476	·0471	·0465	·0460	·0455	·0450	·0445
10	·0440	·0435	·0431	·0426	·0422	·0417	·0413	·0408	·0404	·0400
11	·0396	·0392	·0389	·0385	·0381	·0377	·0373	·0370	·0367	·0363
12	·0360	·0357	·0353	·0350	·0347	·0344	·0341	·0338	·0335	·0332
13	·0329	·0326	·0324	·0321	·0318	·0316	·0313	·0310	·0308	·0305
14	·0303	·0301	·0298	·0296	·0293	·0291	·0289	·0287	·0284	·0282
15	·0280	·0278	·0276	·0274	·0272	·0270	·0268	·0266	·0264	·0262
16	·0260	·0258	·0256	·0254	·0253	·0251	·0249	·0248	·0246	·0244
17	·0242	·0241	·0239	·0237	·0236	·0234	·0233	·0231	·0230	·0228
18	·0227	·0225	·0224	·0222	·0221	·0220	·0218	·0217	·0215	·0214
19	·0213	·0211	·0210	·0209	·0207	·0206	·0205	·0204	·0203	·0201
20	·0200	·0199	·0198	·0197	·0196	·0194	·0193	·0192	·0191	·0190
21	·0189	·0188	·0186	·0185	·0184	·0183	·0182	·0181	·0180	·0179
22	·0178	·0177	·0176	·0175	·0174	·0173	·0172	·0171	·0170	·0170
23	·0169	·0168	·0167	·0166	·0165	·0164	·0163	·0163	·0162	·0161
24	·0160	·0159	·0158	·0158	·0157	·0156	·0155	·0154	·0153	·0152
25	·0152	·0151	·0151	·0150	·0149	·0148	·0147	·0147	·0146	·0145
26	·0145	·0144	·0143	·0143	·0142	·0141	·0140	·0140	·0139	·0138
27	·0138	·0137	·0137	·0136	·0135	·0135	·0134	·0134	·0133	·0132
28	·0132	·0131	·0130	·0130	·0129	·0128	·0128	·0127	·0127	·0126
29	·0126	·0125	·0124	·0124	·0123	·0123	·0122	·0122	·0121	·0121
30	·0120	·0119	·0119	·0118	·0118	·0117	·0117	·0116	·0116	·0115
31	·0115	·0114	·0114	·0113	·0113	·0112	·0112	·0111	·0111	·0110
32	·0110	·0110	·0109	·0109	·0108	·0108	·0107	·0107	·0106	·0106
33	·0105	·0105	·0105	·0104	·0104	·0103	·0103	·0102	·0102	·0102
34	·0101	·0101	·0100	·0100	·0100	·0099	·0099	·0098	·0098	·0098
35	·0097	·0097	·0096	·0096	·0096	·0095	·0095	·0095	·0094	·0094
36	·0093	·0093	·0093	·0092	·0092	·0092	·0091	·0091	·0090	·0090

blank values represent the serum inorganic P. To estimate the P take 5 ml. of each of the filtrates and run in while shaking 4 ml. molybdic acid solution. Then add, as rapidly as possible, 1 ml. dilute stannous chloride solution. Shake immediately. For standard, take 5 ml. diluted standard and treat as above. Read after 15 minutes.

To correct for the interference of trichloracetic acid in the blanks and for the interference of trichloracetic acid and glycerophosphate in the incubated samples the following corrections have to be made :

Blanks.—Look up the values in the table for the reading found and add 5 % of the value. Result × 200 = mg. P per 100 ml. serum.

Incubated samples.—Add 10 % to the value found in the table for the reading, multiply by 400, subtract the value found in the blank, and the result is expressed as mg. P liberated in 1 hour by 100 ml. serum. If, on account of the high phosphate liberation as shown by the colour development, the analysis is repeated, using less than 5 ml. of the filtrate and compensating by the addition of distilled water, then the 10 % correction must be proportionately reduced.

Samples of serum show an increased value for serum phosphatase on standing at laboratory temperature. Therefore this estimation should be carried out as soon as possible after the blood is drawn. By the technique reported for obtaining serum we find it possible to set up the tubes for batches of six samples at a time and to have them in the incubator within one hour of drawing the blood sample.

Note from Oversea Worker.

Robinson, C. H. (Canada). Recommends the method of King and Armstrong (*Canad. Med. Assoc. J.*, 1934, **31,** 376) as an alternative. (This method has been studied in detail by Folley, S. J. and Kay, H. D., *Biochem. J.*, 1935, **29,** 1837.—*Ed.*)

PROTEIN PRECIPITATION FROM OXALATED BLOOD (FOLIN-WU METHOD).

References.

Folin, O. and Wu, H., *J. Biol. Chem.*, 1919, **38,** 81.
Folin, O., *J. Biol. Chem.*, 1922, **51,** 419.

Reagents.

Sulphuric acid : 0·67 *N*.
Sodium tungstate : 10 % solution.

Method.

To 7 ml. water add 1 ml. oxalated blood and 1 ml. 10 % sodium tungstate and mix well. Run in while shaking 1 ml. 0·67 *N* H_2SO_4, stopper and shake vigorously. The mixture should be chocolate brown, and show no foam or bubbles when shaken. If these conditions are not found, the sample can be saved by adding a drop or so of 2*N* H_2SO_4. Too much oxalate or citrate interferes with the precipitation.

Folin, in his second paper, emphasises the importance of checking the purity of the sodium tungstate, *e.g.* for presence of carbonates.

ESTIMATION OF BLOOD CHLORIDES.

Reference.

Whitehorn, J. C., *J. Biol. Chem.*, 1920–21, **45,** 449.

Reagents.

Silver nitrate :	4·791 g. per litre.
Thiocyanate solution :	Dissolve 2·5 g. ammonium thiocyanate (or 3 g. potassium thiocyanate) in 1 litre of water, and adjust so that 5 ml. is equivalent to 5 ml. of the silver nitrate solution.
Ferric indicator :	Dissolve 100 g. iron alum in 1 litre of water. Then add 1 litre conc. HNO_3 and boil the solution to expel oxides of nitrogen.

Method.

Take 10 ml. of the Folin-Wu, protein-free filtrate (*q.v.*), and add 5 ml. silver nitrate solution. Mix and add 5 ml. conc. HNO_3. Mix again and filter through a double Whatman No. 40 filter paper at the end of 5 minutes. Take 10 ml. of the filtrate in a porcelain basin, add 2 ml. ferric indicator and titrate with the thiocyanate solution until a faint salmon-pink colour just persists. Stir well throughout the titration.

Calculation :

100 × [5·0 — (2 × titration value)] = mg. Cl per 100 ml. blood.

At no time after the silver nitrate has been added should the solutions be allowed to stand. The estimation should be proceeded with and completed as quickly as possible.

ESTIMATION OF BLOOD SUGAR (TOTAL REDUCING SUBSTANCES). (HAGEDORN AND JENSEN).

Reference.

Höst, H. F. and Hatlehol, R., *J. Biol. Chem.*, 1920, **42**, 347.

Reagents.

Zinc sulphate :	0·45 % aqueous solution.
Potassium ferricyanide :	Crystals of the salt are washed free from dust and dried in an oven. 1·6490 g. of these crystals and 28·6 g. of crystalline sodium carbonate are dissolved in water and made up to 1 litre.
Potassium iodide solution :	In 200 ml. water dissolve 50 g. NaCl, then 5 g. KI and finally 10 g. neutral zinc sulphate. Add a few crystals of sodium carbonate, shake and filter. This reagent will not keep beyond one week.
Acetic acid :	3 % solution.
Sodium thiosulphate :	$N/10$. Dilute this to $N/200$ as required.
Starch indicator :	Approx. 1 %.

Method.

In a small test tube place 5 ml. of the 0·45 % zinc sulphate solution. With a 0·1 ml. pipette measure exactly 0·1 ml. oxalated blood and blow it into the zinc sulphate solution in order to disperse it throughout the solution. Immediate complete dispersion is essential. Rinse out the pipette several times with the mixture. Place in boiling water for exactly 4 minutes, cool and filter with slight suction through a 5·5 cm. No. 40 Whatman filter paper, receiving the filtrate in a 6″ × 1″ boiling tube. Rinse the small tube with three successive portions of 5 ml. water, washing the precipitate on the filter paper with these washings. To each tube and to two blanks containing 20 ml. distilled water add 2 ml. potassium ferricyanide solution and mix. Heat in a boiling water-bath for exactly 15 minutes and cool. Then add 3 ml. of the KI solution and 2 ml. 3 % acetic acid. Titrate with the $N/200$ thiosulphate until the yellow just fades, add 3 drops of starch indicator, and titrate to the end point.

The blank titration should not require less than 1·97 ml. of $N/200$ thiosulphate.

Calculation :

Blank titration — titration value = reduction value.

Reduction Value ml. *N*/200 Thiosulphate.	Mg. Total Reducing Substances per 100 ml. Blood.
0·25	44·2
0·30	53·0
0·35	61·8
0·40	70·6
0·45	79·4
0·50	88·2
0·55	97·0
0·60	105·8
0·65	114·6
0·70	123·4
0·75	132·2

Note by Oversea Worker.

Aykroyd (India). Uses a micro-method of Sankaran and Rajagopal (*Indian J. Med. Res.*, 1936, **24,** 459) which is a modification of the method of Shaffer and Williams (*J. Biol. Chem.*, 1935, **111,** 707).

ESTIMATION OF BLOOD SUGAR. (SHAFFER-HARTMANN METHOD—SOMOGYI'S MODIFICATION.)

References.

Shaffer, P. A. and Hartmann, A. F., *J. Biol. Chem.*, 1920–21, **45,** 365.
Somogyi, M., *J. Biol. Chem.*, 1926, **70,** 599.

Reagents.

Tartrate-carbonate-copper reagent :

Copper sulphate (cryst.)	6·5 g.
Rochelle salt	12·0 g.
Sodium carbonate (anhyd.)	20·0 g.
Sodium bicarbonate	25·0 g.
Potassium iodide	10·0 g.
Potassium iodate	0·8 g.
Potassium oxalate	18·0 g.

Dissolve the Rochelle salt, sodium carbonate and sodium bicarbonate in about 500 ml. water, and into this pour with stirring the copper sulphate dissolved in about 100 ml. water. Then add the solution of the other constituents and dilute to 1 litre. (*N.B.*—The potassium iodate must be weighed quite accurately.)

Sulphuric acid : *N* solution.
Sodium thiosulphate : 0·005 *N* (see Hagedorn-Jensen method).

Method.

Measure 5 ml. of the tartrate-carbonate-copper reagent into a large test tube (10″ × 1″) or into a sugar flask (round-bottomed, short-necked, 200 ml.), and add 5 ml. of the protein-free, Folin-Wu filtrate

AMOUNTS OF GLUCOSE CORRESPONDING TO TITRATION VALUES WHEN 5 ml. OF 1 : 10 BLOOD FILTRATE AND 5 ml. OF COPPER REAGENT ARE HEATED IN A WATER-BATH FOR 15 MINUTES.

ml. *N*/200 thiosulphate.	Tenths of 1 ml. *N*/200 sodium thiosulphate.									
	0	1	2	3	4	5	6	7	8	9
	Mg. glucose in 100 ml. blood.									
0			21	23	26	29	31	34	36	39
1	41	44	46	49	51	53	56	58	61	63
2	65	68	70	72	75	77	80	82	84	86
3	89	92	94	97	99	101	103	106	108	110
4	113	115	117	119	121	124	126	128	130	132
5	135	137	139	141	143	146	148	150	152	154
6	157	159	161	163	165	168	170	172	174	176
7	179	181	183	185	187	190	192	194	196	199
8	201	203	205	207	210	212	214	216	218	221
9	223	225	227	230	232	234	237	239	241	243
10	245	248	250	252	254	256	259	261	263	265
11	267	270	272	274	276	279	281	283	285	288
12	290	292	294	296	299	301	303	305	308	310
13	312	314	316	318	321	323	326	328	330	332
14	334	337	339	341	343	345	347	350	352	354
15	356	359	361	363	365	367	370	372	374	376
16	378	381	383	386	388	390	392	394	396	398
17	400									

(see protein precipitation). Mix by gentle shaking and close the tube or flask with a loosely-fitting, pear-shaped glass bulb, and set the tube or flask in a boiling water-bath for exactly 15 minutes. Then cool to 35–40° C., add with agitation from a quick delivery pipette 5 ml. *N* H_2SO_4, seeing that all the copper oxide is promptly dissolved. After about 2 minutes titrate with *N*/200 thiosulphate. A blank titration is performed on 5 ml. of the reagent after heating it as above with 5 ml. water. The difference between the two titrations represents the sugar in the blood, which may be read from the table on page 54.

These directions are applicable when 5 ml. of the protein-free filtrate contain 0·1–2·0 mg. glucose. For higher concentrations the blood filtrates are diluted or a smaller volume, plus the water necessary to make up to 5 ml., is taken for the determination.

The conditions as to heating, etc., must be strictly adhered to.

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